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Pleiotropic functions of TNF-α determine distinct IKKβ-dependent hepatocellular fates in response to LPS

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[1]*Department of Anatomy and Cell Biology,* [2]*Department of Internal Medicine-Division of Pulmonary and Critical Care,* [3]*Center for Gene Therapy at the University of Iowa College of Medicine, Iowa City, Iowa; and* [4]*Human Gene Therapy, Faculty of Medicine at the Akdeniz University, Antalya, Turkey*

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Dajani, Rana, Salih Sanlioglu, Yulong Zhang, Qiang Li, Martha M. Monick, Eric Lazartigues, Timothy Eggleston, Robin L. Davisson, Gary W. Hunninghake, and John F. Engelhardt. Pleiotropic functions of TNF-α determine distinct IKKβ-dependent hepatocellular fates in response to LPS. *Am J Physiol Gastrointest Liver Physiol* 292: G242–G252, 2007. First published August 24, 2006; doi:10.1152/ajpgi.00043.2006.—TNF-α influences morbidity and mortality during the course of endotoxemia. However, the complex pleiotropic functions of TNF-α remain poorly understood. We evaluated how hepatic induction of NF-κB and TNF-α influence survival and hepatocellular death in a lethal murine model of endotoxic shock. Using dominant-negative viral vectors to inhibit the IKK complex, we demonstrate through this study that the liver is a major source of TNF-α during the course of lethal endotoxemia and that IKKβ (but not IKKα) is predominantly responsible for activating NF-κB and TNF-α in the liver after LPS administration. Using TNF-α knockout mice and hepatic-specific inhibition of IKKβ, we demonstrate that the status of TNF-α and NF-κB balances necrotic and apoptotic fates of hepatocytes in the setting of endotoxemia. In the presence of TNF-α, inhibiting hepatic IKKβ resulted in increased survival, reduced serum proinflammatory cytokines, and reduced hepatocyte necrosis in response to a lethal dose of endotoxin. In contrast, inhibiting hepatic IKKβ in TNF-α knockout mice resulted in decreased survival and increased caspase 3-mediated hepatocyte apoptosis after endotoxin challenge, despite a reduced proinflammatory cytokine response. In the presence of TNF-α, NF-κB-dependent hepatocellular necrosis predominated, while in the absence of TNF-α, NF-κB primarily influenced apoptotic fate of hepatocytes. Changes in JNK phosphorylation after LPS challenge were also dynamically affected by both IKKβ and TNF-α; however, this pathway could not solely explain the differential outcomes in hepatocellular fates. In conclusion, our studies demonstrate that induction of NF-κB and TNF-α balances protective (antiapoptotic) and detrimental (proinflammatory) pathways to determine hepatocellular fates during endotoxemia.

nuclear factor-κB; endotoxic shock; inflammation; apoptosis; c-jun NH_2-terminal kinase

LPS IS a component of the gram-negative bacterial cell wall that plays a major role in the pathogenesis of the sepsis syndrome (11). During the course of sepsis, the production of proinflammatory cytokines such as TNF-α leads to systemic inflammatory response syndrome (29). Moreover, TNF-α has been shown to be a major player in endotoxin-induced lethality during the course of sepsis (3). Numerous animal studies have been conducted in an effort to understand the pathophysiology of sepsis. For example, mice lacking TNF-α or TNF receptor 1 are resistant to endotoxin (33, 34). In addition, neutralizing antibodies to TNF-α or soluble recombinant TNF receptor, protect against LPS injury (1, 22, 40). These studies suggested that downregulation of TNF-α production, or its neutralization, might prove useful in the treatment of sepsis.

LPS induction of TNF-α is largely dependent on NF-κB activation (38). NF-κB nuclear translocation is regulated by two IκB kinases: IKKα (IKK-1) and IKKβ (IKK-2) (30, 46). Although a direct link between TNF-α induction and endotoxin-induced cell death has been clearly established, the in vivo role of NF-κB in producing a proinflammatory state during endotoxemia, while at the same time modulating cell survival, has remained unclear. For example, although NF-κB is known to induce TNF-α gene expression after LPS exposure (38), NF-κB activity has also been shown to be an important antiapoptotic factor during liver development and regeneration (19, 23, 24). Similarly, reports have suggested that TNF-α-induced apoptosis can be abrogated through NF-κB activation (42). Hence, the manner by which NF-κB balances the induction of proinflammatory and antiapoptotic pathways remains ambiguous.

Using in vivo liver-directed gene transfer techniques, we have evaluated whether hepatic-derived NF-κB-regulated TNF-α production is an important pathophysiologically relevant component in endotoxemic death. Results from these studies suggest that hepatic IKKβ, but not IKKα, plays a predominant role in NF-κB activation and the subsequent rise in serum TNF-α levels during the course of lethal endotoxemia. Inhibition of IKKβ using a dominant-negative adenoviral vector resulted in increased survival, reduced serum TNF-α, and reduced liver injury in response to a lethal dose of endotoxin. Such findings suggest that during the course of lethal endotoxemia, NF-κB activation has a predominantly negative proinflammatory effect on the liver through the induction of TNF-α. In contrast, in TNF-α-knockout mice, inhibition of IKKβ decreased survival and increased liver apoptosis after LPS challenge. These data suggest that during the course of lethal endotoxemia, TNF-α influences the role NF-κB as an antiapoptotic factor in the liver.

MATERIALS AND METHODS

Recombinant adenovirus vectors and infections. For functional studies, the following recombinant adenoviral vectors were used: *1*) a

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dominant-negative mutant (K44M) of IKKα (Ad.IKKαKM) (36); *2*) a dominant-negative mutant (K44A) of IKKβ (Ad.IKKβKA) (36); and *3*) a dominant-negative mutant (S32/36A) of IKBα (Ad.IKBαS32/36A) (19). An adenovirally encoded luciferase reporter gene driven by an NF-κB responsive promoter (Ad.NFkBLuc) was used for transcriptional reporter assays (36). Two control recombinant adenoviral vectors were used and gave similar results (Ad.EGFP and Ad.BglII, an empty vector control). Recombinant adenoviral stocks were generated as previously described (13). Viral infections were performed by tail-vein injection of 10^{11} particles of purified virus in 100 μl of saline 2 wk before experiments. A nu/nu athymic mouse background was used both to avoid potential complications of cellular immunity to adenoviral vectors and because hepatic transgene expression is stable for long periods of time (up to 125 days) on this nu/nu background (45). A 2-wk waiting period after infection was used to allow time for acute effects of adenoviral infection to subside.

Endotoxemia model. All animal experimentation was performed in accordance with the principles and procedures outlined in the National Institutes of Health guidelines for the care and use of experimental animals. Male (~30 g) athymic nu/nu mice (Harlan Sprague Dawley) were used for all studies. For the studies in TNF-α knockout mice, we bred the TNF-$\alpha^{-/-}$ knockout allele from B6, 129S-Tnftm1Gkl mice (Jackson ImmunoResearch Laboratories, West Grove, PA) onto the nu/nu background. Studies evaluating the contribution of TNF-α on the nu/nu background were always performed with matched TNF-$\alpha^{+/-}$ nu/nu and TNF-$\alpha^{-/-}$ nu/nu littermates. LPS isolated from *Escherichia coli* serotype 055:B5(L-2880) (Sigma, St. Louis, MO) was used to induce lethal endotoxemia. LPS was dissolved in PBS and injected intraperitoneally into mice (40 μg/g body wt for the nu/nu background and 20 μg/g body weight for nu/nu:TNF-α fixed background). The hypodynamic phase of endotoxic shock was confirmed by monitoring MAP (15). Carotid arterial catheters were placed 24 h before experiments were initiated and MAP was measured in conscious mice. MAP was continuously recorded beginning 30-min before and up to 24 h post-LPS treatment, as described previously (9); partial hepatectomy (PH) was performed as described elsewhere (19).

Serum measurements and NF-κB transcriptional assays. Sera were collected by retroorbital bleeding of mice at different time points post-LPS administration. Serum TNF-α levels were measured using a DuoSet ELISA Kit from R&D Systems (Minneapolis, MN). Serum alanine aminotransferase (ALT) measurements (an indication of liver damage) were performed, as previously described (48). All other serum cytokines were measured in a simultaneous, multiplexed format, using a micro-bead and flow-based protein detection system (Bio-Plex System, Bio-Rad Laboratories, Hercules, CA), based on the Luminex MAP technology, as previously described (17). A nonpaired *t*-test was used for statistical analysis with $P < 0.05$ as significant. In vivo NF-κB transcriptional assays using Ad.NFκBluc virus were performed as follows. Ad.NFκBLuc virus was coinjected with recombinant adenoviral constructs expressing IKKαKM or IKKβKA into mice through the tail vein. Similar control infections were performed with Ad.NFκBluc and Ad.Control vectors. Two weeks after infection, mice received intraperitoneal injections of LPS at the concentration of 20 or 40 μg/g body wt. At 2 h post-LPS administration, animals were anesthetized with ketamine/xylazine, followed by intracardiac perfusion with 1 × PBS before harvesting the liver. A portion of liver tissue was homogenized in 1 ml of 1 × cell culture lysis buffer (Promega, Madison, WI) supplemented with 1 tablet of protease inhibitor cocktail from Promega The Luciferase Assay System with Reporter Lysis Buffer (Promega, Catalog No. E4030) was used to measure NF-κB-mediated transcriptional induction according to the manufacturer's protocol. All measurements of luciferase activity (relative light units) were normalized to the protein concentration using a Bradford assay.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assays and electron microscopy. For terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining, livers were perfused with PBS and embedded in optimal cutting temperature compound (OCT) by quick freezing. An in situ cell death detection kit (TMR red, Roche) was used to detect apoptotic cells in liver sections, and results were analyzed by fluorescent microscopy. Electron and bright-field microscopic analysis of hepatic necrosis and apoptosis were performed as followed. Mouse livers were perfused with a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer pH 7.2 followed by dissection in the fixative. After three 0.1 M sodium cacodylate buffer rinses, samples were exposed to a solution of 1% osmium tetroxide, 1.5% potassium ferrocyanide and 0.1 M sodium cacodylate for 1 h. After subsequent buffer rinses, en bloc staining with 2.5% uranyl acetate was followed by dehydration through a graded ethanol series. Transmission electron microscope (TEM) samples were infiltrated with Eponate 12 epoxy resin (Ted Pella, Redding, CA), and sectioning was performed with Leica UC6 ultramicrotome. One-hundred-nanometer sections were postsection-stained with uranyl acetate and Reynold's lead citrate for TEM analysis. TEM images were recorded using a JEOL JEM-1230 TEM equipped with a Gatan Ultrascan 1000 CCD camera. For bright-field microscopy, mouse livers were perfused with PBS before embedding in OCT compound by quick freezing (Tissue-Tek, Elkhart, IN). Ten-micrometer-thick sections were stained with standard hematoxylin and eosin stain.

Western Blot analysis. The Odyssey infrared-imaging system (Lycor) was used for Western blot analysis of liver lysates and quantification of specific protein. Antibodies and company sources were Anti-HA peroxidase antibody (Roche Molecular Diagnostic, Indianapolis, IN) was used to detect expression from Ad.IKKαKM and Ad.IKKβKA vectors. The following additional primary antibodies and sources are as follows: anti-GADD45β (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-cleaved caspase 3 (Cell Signaling, Beverly, MA), anti-phospho-JNK1 (Santa Cruz Biotechnologies), anti-JNK1 (Santa Cruz Biotechnologies), anti-cIAP2 (Abcam, Cambridge, MA), and anti-Actin (Santa Cruz Biotechnologies).

Assessment of hepatic IKK kinase activity. Mice were infected with Ad.IKKαKM, Ad.IKKβKA, or Ad.EGFP 2 wk before challenging with LPS. At 0, 30, and 60 min after LPS administration, liver samples from each animal group were harvested and homogenized in preparation for radioactive IKK kinase assays, as previously described (14). Samples were spun at 5,000 *g* for 5 min, and the IKK kinase complex was immunoprecipitated from the cleared supernatant using anti-IKK antibody and protein-A agarose beads. The precipitant was then mixed with 1 μg substrate protein (GST-IκBα), 0.3 mM cold ATP and 10 μCi [γ–^{32}P] ATP in 10-μl kinase buffer (40 mM HEPES, 1 mM β-glycerophosphate, 1 mM nitrophenolphosphate, 1 mM Na_3VO_4, 10 mM $MgCl_2$, and 2 mM DTT), and incubated at 30°C for 30 min. The reaction was terminated on ice and separated by SDS-PAGE followed by transfer to nitrocellulose membrane and autoradiography. Following autoradiography, membranes were then Western blotted with anti-GST antibody to confirm equal loading of the GST-IκBα substrate.

RESULTS

LPS-mediated NF-κB activation is inhibited, and survival is increased by ectopic expression of a dominant negative IKKβ mutant. With an overall goal to evaluate whether hepatic NF-κB induction during endotoxemia plays a deleterious or protective role in survival, we sought to establish the effectiveness of inhibiting NF-κB activation through the IKK complex in vivo. Two recombinant adenoviral vectors expressing dominant-negative forms of IKKα (Ad.IKKαKM) or IKKβ (Ad.IKKβKA) were used for these studies (36). Western blot analysis of liver lysates from Ad.IKKαKM- and Ad.IKKβKA-infected animals indicated that both transgenes were expressed at equal levels in the liver (Fig. 1*A*). These mice demonstrated

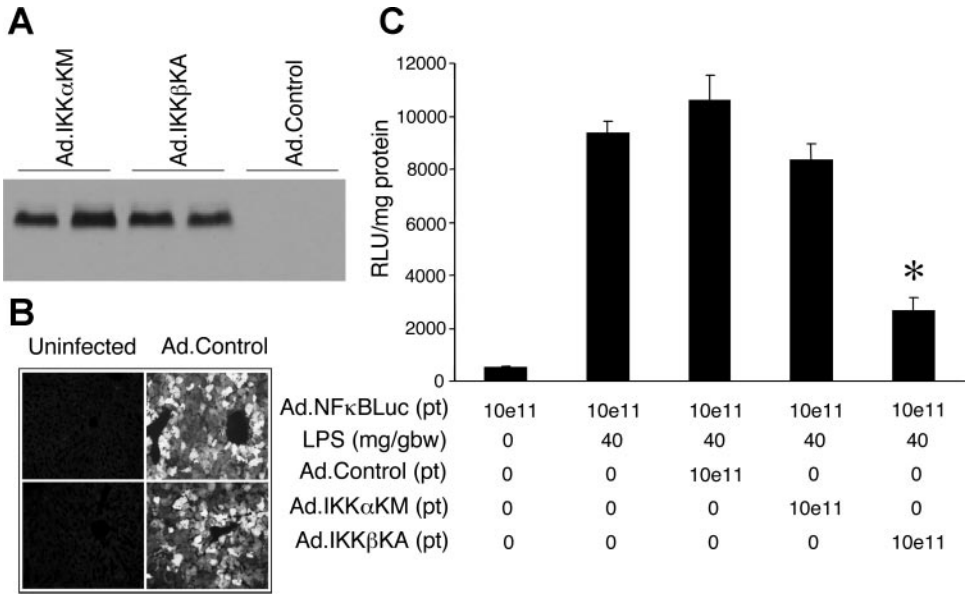

Fig. 1. Dominant mutant of IKKβ inhibits NF-κB activation in endotoxemic mice. Mice were injected with 1×10^{11} particles (pt) of Ad.Control, Ad.IKKαKM, or Ad.IKKβKA virus through the tail vein. Two weeks after infection, livers were harvested and examined for transgene expression, or animals were used in LPS experiments. *A*: 30 μg of liver protein extract were loaded in each lane and IKK expression was detected by Western blot with an anti-HA peroxidase-labeled antibody (duplicate independent liver samples are shown). IKK proteins were tagged with the hemagglutinin (HA) amino acid sequence. *B*: fluorescent micrographs of uninfected and Ad.EGFP infected livers (two independent examples are given, and exposure times were equivalent for all panels). *C*: LPS-induced NF-κB transcription activation was evaluated in livers coinfected with Ad.NFκBLuc and Ad.Control, Ad.IKKαKM, or Ad.IKKβKA. Two weeks after infection, mice were injected with LPS [40 μg/g body weight (gbw)], and livers were harvested and assayed for NF-κB activity 2 h after LPS injection, as described in MATERIALS AND METHODS. Experimental conditions are provided on the *x*-axis. The mean ± SE relative luciferase activity is indicated as relative light units (RLU) on the *y*-axis for $n = 4$ animals in each experimental condition. ANOVA followed by Bonferroni's multiple comparison test was used to assess statistical analysis with $P < 0.05$ as significant. *Statistically significant differences ($P < 0.001$) between the three other LPS-challenged groups. Ad.Control- and Ad.IKKαKM-infected groups were not significantly different from the Ad.NF-κBluc-infected LPS-treated animals.

stable high-level transgene expression in the majority of hepatocytes 2 wk after tail vein infection (Fig. 1*B*). To assess the functional ability of the Ad.IKKαKM and Ad.IKKβKA constructs to inhibit LPS-mediated NF-κB activation, mice were coinfected with an adenovirus vector carrying a luciferase reporter gene under the control of NF-κB regulatory sites (Ad.NFκBLuc) (36). Only Ad.IKKβKA infection significantly reduced LPS-induced NF-κB activity post-LPS exposure (Fig. 1*C*). No reduction was obtained when animals were infected with Ad.Control or Ad.IKKαKM. These results suggested that NF-κB transcriptional activation in the liver by LPS is predominantly controlled by IKKβ, a finding consistent with data in cell lines (36).

After establishing the parameters for efficient gene delivery of IKK mutants to the liver, the effects of hepatic IKKαKM or IKKβKA expression on survival in this endotoxemia model were evaluated following a lethal dose of LPS. Death in this model was clinically similar to the hypodynamic phase of sepsis (5, 15). Mean arterial pressure (MAP) of nude mice decreased from 104 ± 5.4 mmHg to 65 ± 3 mmHg 24 h post-LPS treatment (Fig. 2*A*). However, the reduction in MAP was significant as early as 3 h following LPS administration. Ad.IKKαKM or Ad.Control infection did not alter the survival curve following LPS challenge over the course of 12 days (Fig. 2*B*). In contrast, Ad.IKKβKA administration significantly improved the survival rate by 25% at 12 days following LPS injection. These data demonstrate that IKKβKA expression in the liver decreases LPS-induced lethality in nude mice.

Previous studies have shown that serum TNF-α levels correlate with the severity of sepsis in humans (29). To account for the therapeutic effect of hepatic IKKβKA expression and the extent of liver injury, serum TNF-α and ALT levels were analyzed post-LPS challenge. Maximum elevation in TNF-α and ALT levels was detected 3 h post-LPS challenge (Fig. 3, *A* and *B*). The expression of IKKβKA, but not IKKαKM, dramatically reduced LPS-induced TNF-α and ALT levels in these mice (Fig. 3, *A* and *B*). This reduction correlated with a decrease in LPS-mediated NF-κB activation (Fig. 1*C*). These results demonstrate that NF-κB activation is detrimental to the liver after LPS challenge. However, these findings contradict previous reports demonstrating that NF-κB plays a major protective, antiapoptotic, role during hepatic development and regeneration following PH (19, 23). Previous studies have demonstrated that recombinant adenovirally mediated expression of the IκBαS32/36A mutant (which blocks NF-κB activation) enhances hepatocellular apoptosis leading to death following a 2/3 PH in adult rats (19). To ensure that the differing functional outcomes in these two hepatic models (endotoxemia and PH) were not a result of vector-specific effects, we evaluated the ability of hepatic IKKβKA expression to modulate survival in a 2/3 PH model. In head-to-head experiments, we observed that adenoviral-mediated expression of IKKβKA or IκBαS32/36A similarly reduced survival following a 2/3 PH ($P < 0.001$) compared with control vector infection (data not shown). These findings suggest that NF-κB has context-specific roles following PH and endotoxemia that affect hepatocyte survival differently. Interestingly, Ad.IKKαKM-infected animals had similar survival rates as control vector-infected animals following 2/3 PH (data not

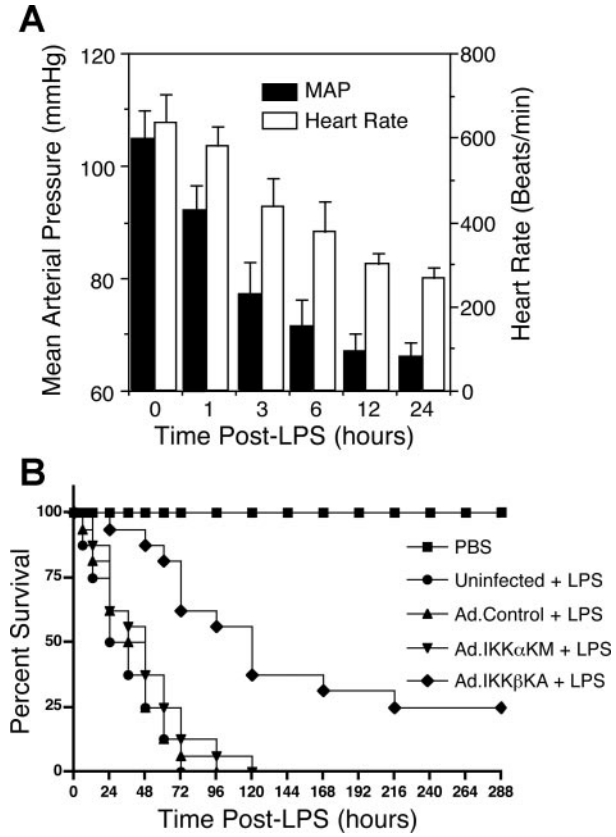

Fig. 2. IKKβKA expression in the liver increases survival after experimentally induced lethal endotoxic shock. *A*: criteria for the hypodynamic phase of sepsis were established by monitoring mean arterial pressure (MAP) after LPS administration. Carotid arterial catheters were placed 24 h before experiments were initiated, and MAP was measured in conscious mice. MAP was continuously recorded beginning 30 min before, and up to 24-h after, LPS treatment, as described in the MATERIALS AND METHODS ($n = 3$). Statistical differences in MAP post-LPS were confirmed using ANOVA followed by Dunnett's multiple comparison tests. *B*: nude mice were either mock infected with PBS or infected with either Ad.Control, Ad.IKKαKM, or Ad.IKKβKA constructs, 2 wk before IP LPS challenge at 40 μg/gbw. The survival was monitored every 12 h after LPS administration for a total of 12 days. Each curve represents the data obtained from 16 animals treated with LPS, with the exception of the PBS controls not treated with LPS ($n = 8$). The *x*-axis represents hours after LPS administration. The survival rate is expressed as the percent survival on the *y*-axis. Statistical analysis of survival curves using the Log rank test indicates that there is a significant difference in the survival rate of Ad.IKKβKA-infected animals ($P < 0.0001$).

shown). Hence, IKKβ appears to play a dominant role in regulating responses to both PH and endotoxemia.

Hepatic expression of IKKβKA, but not IKKαKM, inhibits IKK kinase activation following LPS challenge. To confirm that LPS-induced hepatic activation of the IKK complex was modulated by our dominant negative IKK vectors in a similar fashion to NF-κB activity (Fig. 1*C*), we performed IKK kinase assays. IKK kinase activation was evaluated in liver lysates following LPS challenge from three groups of animals (Ad.IKKβKA-, Ad.IKKαKM-, and Ad.Control-infected). Previous studies in immune-competent mice have demonstrated that IKK kinase activity in the liver increases by 30 min after LPS challenge, with sustained activation out to 1 h (14). Our studies in Ad.Control-infected nu/nu animals demonstrated this same pattern of LPS-induced IKK kinase activation (30–60 min), as indicated by the ability of immunoprecipitated IKK kinase complex to phosphorylate GST-IκBα in an in vitro kinase assay (Fig. 4, *A* and *B*). Hepatic expression of IKKαKM did not significantly alter the pattern of IKK kinase activation following LPS challenge (Fig. 4*A*). In contrast, hepatic expression of IKKβKA significantly inhibited IKK kinase complex activation at 30–60 min after LPS challenge (Fig. 4*B*). Quantitative assessment of IKKβKA-mediated inhibition of IKK kinase activity demonstrated a statistically significant 4- and 2.5-fold reduction in IKK kinase activity at 30 and 60 min post-LPS challenge, respectively, compared with control vector-treated animals. This level of inhibition by IKKβKA was extremely similar to that seen for LPS-mediated NF-κB transcriptional activation at 2 h after injury (Fig. 1*C*).

Hepatic IKKβKA expression in TNF-α-knockout mice results in decreased survival post-LPS challenge. We used TNF-α-knockout mice to investigate whether the observed deleterious nature of NF-κB activation post-LPS challenge was solely dependent on TNF-α induction by NF-κB. As previously reported, TNF-α-deficient animals were resistant to LPS-induced death compared with their heterozygous TNF-$\alpha^{+/-}$ littermates (33). This resistance was not altered on the nu/nu background following infection of the liver with control recombinant adenovirus (Fig. 5). Ad.Control-infected TNF-$\alpha^{+/-}$ nu/nu mice all died by 36 h after LPS challenge, while all Ad.Control-infected TNF$\alpha^{-/-}$ nu/nu littermates survived. Fur-

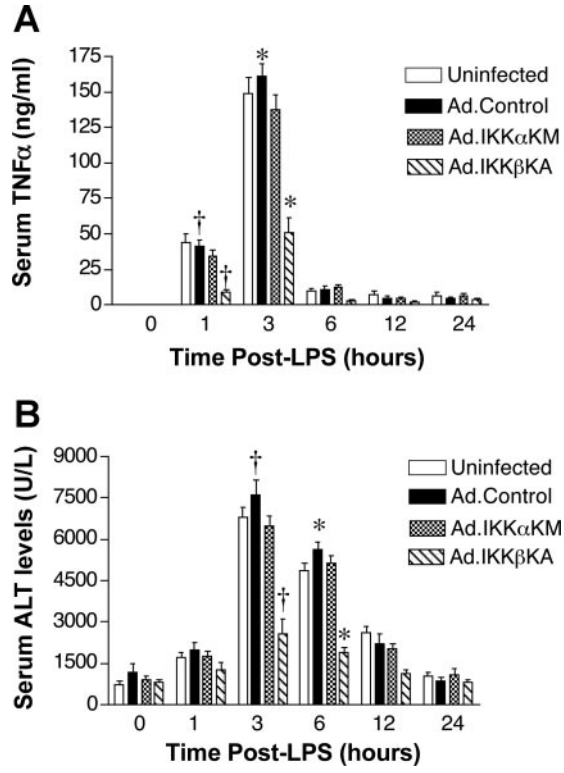

Fig. 3. Hepatic expression of the dominant negative mutant to IKKβ (IKKβKA) decreases LPS-induced serum TNF-α and alanine aminotransferase (ALT) levels after a lethal dose of LPS. Mice were infected with adenoviral constructs, as indicated 2 wk before LPS administration. Sera were collected at different time points after LPS injections, as indicated on the *x*-axis. Serum TNF-α levels (*A*) and serum ALT levels (B) after LPS administration. In *A* and *B*, each bar represents a mean ± SE of $n = 6$ independent animals points (the same animals were evaluated in each graph). †·*Significant different comparisons using the Student's *t*-test ($P < 0.005$).

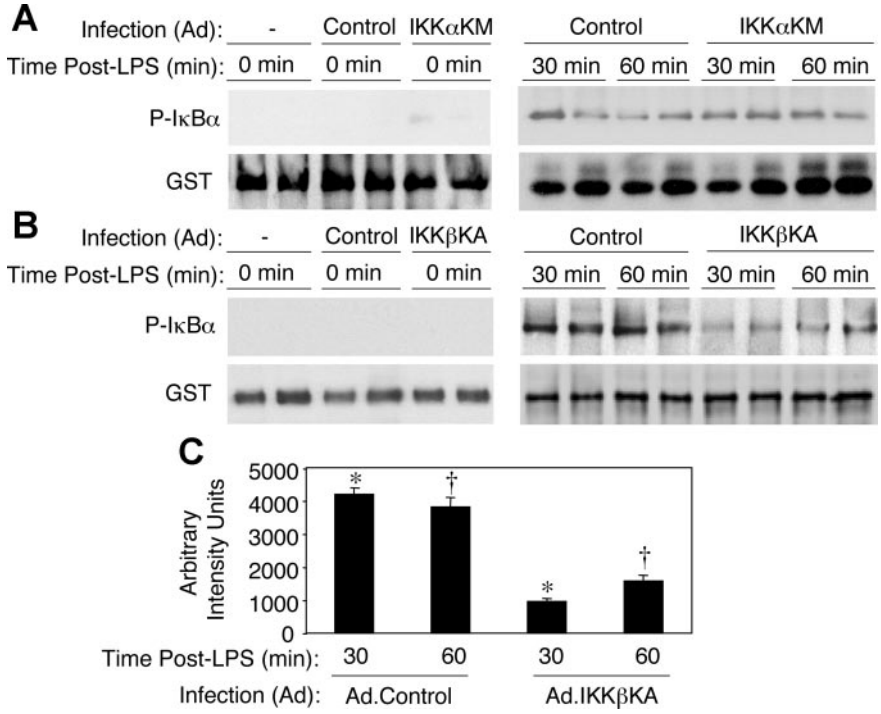

Fig. 4. IKKβKA inhibits the IKK complex in vivo. To assess the extent to which IKKαKM or IKKβKA expression modulates IKK complex activation in the liver after LPS challenge, nude mice were infected with 1×10^{11} particles (pt) of Ad.Control, Ad.IKKαKM, or Ad.IKKβKA virus through the tail vein. Two weeks after infection, animals were challenged with LPS, and livers were harvested at the indicated time points for analysis of IKK kinase activity, as described in MATERIALS AND METHODS. *A*: comparative analysis of Ad.Control vs. Ad.IKKαKM-infected livers with two representative independent animals shown for each time point. *Top*: autoradiograms of the GST-IκBα substrate. *Bottom*: Western blot analysis using anti-GST antibody to control for substrate loading. *B*: comparative analysis of Ad.Control vs. Ad.IKKβKA-infected livers using the same presentation format as in *A*. *C*: quantitation of IKK kinase activity from Ad.Control vs. Ad.IKKβKA-infected livers after LPS challenge. Results depict the means ± SE relative density of phosphorylated GST-IκBα for each condition ($n = 4$ independent animals for each time point). †·*Significant different comparisons using the Student's *t*-test ($P < 0.001$).

thermore, the ability of hepatic IKKβKA expression to increase survival post-LPS challenge was also retained on the TNF$^{+/-}$ nu/nu background (Fig. 5). Interestingly, hepatic Ad.IKKβKA infection of TNF-α$^{-/-}$ nu/nu mice led to a loss of resistance to LPS, demonstrating significantly decreased survival (40%) compared with Ad.Control-infected TNF-α$^{-/-}$ nu/nu littermates (100%). This finding suggested that hepatic IKKβKA expression modulates LPS-induced hepatic injury in a TNF-α-dependent fashion.

Hepatic IKKβKA expression induces hepatocellular apoptosis in a TNF-α-dependent fashion post-LPS challenge. To investigate why hepatic IKKβKA expression specifically induced mortality post-LPS challenge selectively in TNF-α$^{-/-}$ mice, but not in TNF-α$^{+/-}$ mice, we evaluated the extent of hepatic apoptosis. Cleaved caspase-3 was used as an indicator for hepatic apoptosis. Western blots of liver tissue homogenates demonstrated a significant increase in the ratio of cleaved caspase-3 to actin at 12 h post-LPS challenge in Ad.IKKβKA-infected livers of TNF-α$^{-/-}$ mice ($P < 0.05$). This increase was not seen in Ad.Control-infected TNFα$^{-/-}$ animals nor in Ad.IKKβKA or Ad.Control infected TNFα$^{+/-}$ mice exposed to LPS (Fig. 6, *A* and *B*). Differences in the extent of apoptosis between the two experimental conditions that resulted in maximal death after LPS challenge (Ad.IKKβKA−TNF-α$^{-/-}$, Ad.Control−TNF-α$^{+/-}$) were confirmed using the TUNEL assay on liver sections (Fig. 6*C*). Hence, our data suggest that dominant-negative IKKβ induces apoptosis in the liver when TNF-α is absent.

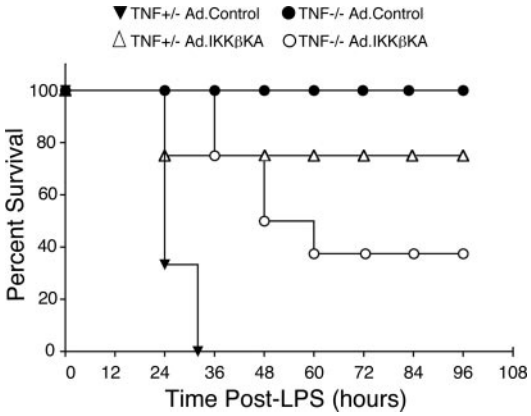

Fig. 5. IKKβKA expression in the liver of TNF-α^{-}/$^{-}$ mice decreases survival after experimentally induced lethal endotoxic shock. TNF-α^{-}/$^{+}$ and TNF-α^{-}/$^{-}$ mice were infected with indicated recombinant adenoviral constructs 2 wk before intraperitoneal injection of LPS at 20 μg/gbw. The survival was monitored every 12 h after LPS administration for a total of 12 days (no animals died past time points plotted). Each curve represents the data obtained from 8 animals. The *x*-axis represents hours after LPS administration. The survival rate is expressed as the percent survival on the *y*-axis.

To determine whether LPS-induced death in TNF-α$^{-/-}$ Ad.IKKβKA- and TNF-α$^{+/-}$Ad.Control-infected mice was due to a different mechanism of hepatic failure, we evaluated hepatic histopathology by light and electron microscopy (Fig. 6, *D* and *E*). Distinct hepatic histopathology was observed in the two experimental groups. Liver sections from TNF-α$^{-/-}$ Ad.IKKβKA-infected LPS-treated mice demonstrated considerable cell shrinkage and condensed cytoplasm indicative of apoptosis, with little or no inflammation (Fig. 6, *D* and *E*). In contrast, liver sections from TNF-α$^{+/-}$Ad.Control-infected mice lacked evidence of condensed cytoplasm or cell shrinkage but had more frequent cellular infiltration, indicative of inflammation (Fig. 6, *D* and *E*). Additionally, nuclear membrane integrity was significantly disrupted in TNF-α$^{+/-}$Ad.Control-infected hepatocytes, consistent with signs of necrosis (Fig. 6*F*). These results are similar to previous findings that have shown that liver failure caused by a lethal dose of LPS without GalN sensitization is characterized by hepatocellular necrosis (4). In contrast, the nuclear membrane remained intact in TNF-α$^{-/-}$Ad.IKKβKA-infected hepatocytes, consistent with

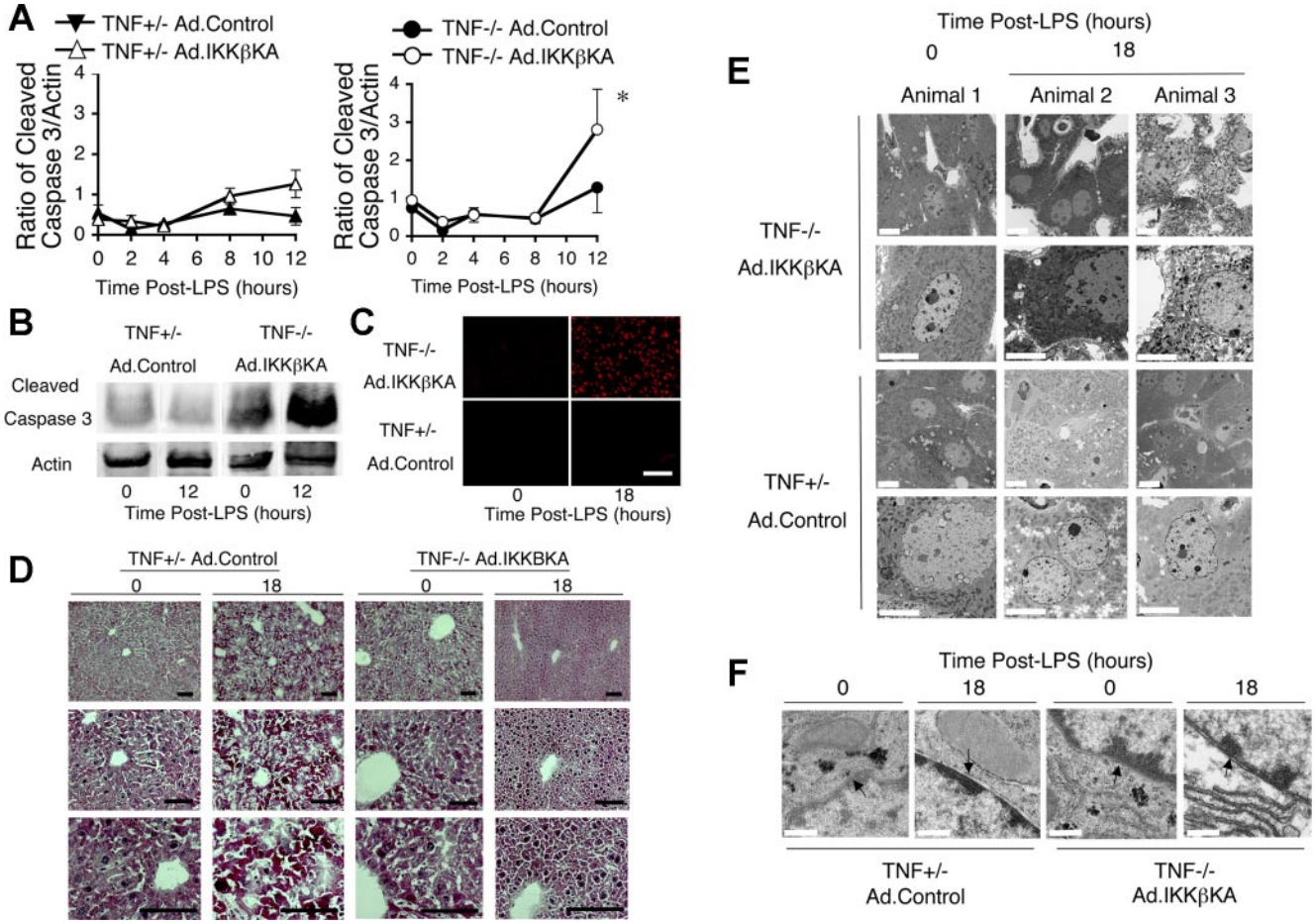

Fig. 6. Ad.IKKβKA infection of the liver of TNF-$\alpha^{-/-}$ mice reduced LPS-induced liver apoptosis. TNF-$\alpha^{+/-}$ or TNF$\alpha^{-/-}$ nu/nu animals were infected with either Ad.Control or Ad.IKKβKA constructs, 2 wk before IP LPS challenge with 20 μg/gbw. *A*: liver tissue was harvested at different time points after LPS injection. The ratio of cleaved caspase 3/actin was calculated by Western blot analysis (means ± SE, $n \geq 3$ independent data points). Unpaired *t*-test was used for statistical analysis with $P < 0.05$ as significant (*), comparing TNF-$\alpha^{-/-}$ Ad.IKKβKA animals with TNF-$\alpha^{+/-}$ Ad.Control-infected animals. *B*: Western blot analysis for hepatic cleaved caspase 3 and actin at 0 and 12 h post-LPS challenge (100 μg was loaded in each lane). *C*: in situ TUNEL analysis for hepatic apoptosis in liver sections from TNF-$\alpha^{+/-}$ Ad.Control- or TNF-$\alpha^{-/-}$ Ad.IKKβKA-infected mice at 0 or 18 h after LPS challenge (scale bar = 0.1 mm). *D*: hematoxylin and eosin-stained bright field images of liver sections from TNF-$\alpha^{+/-}$ Ad.Control- or TNF-$\alpha^{-/-}$ Ad.IKKβKA-infected mice at 0 or 18 h after LPS challenge (scale bar = 0.1 mm). *E*: transmission electron microscope (TEM) images of liver sections from TNF-$\alpha^{+/-}$ Ad.Control or TNF-$\alpha^{-/-}$ Ad.IKKβKA mice at 0 or 18 h after LPS challenge. Two different animals are shown for the 18-h time point (scale bar = 5 μm). *F*: TEM images of liver sections at larger magnification showing nuclear membranes (scale bar = 400 nm).

apoptosis (Fig. 6*F*). These results support the hypothesis that LPS-induced death in TNF-$\alpha^{-/-}$Ad.IKKβKA- and TNF$\alpha^{+/-}$ Ad.Control-infected mice was due to different mechanisms of hepatic failure.

Hepatic IKKβKA expression reduces LPS-induced liver inflammation in a TNF-α-dependent fashion. Histopathology suggested that in the presence of TNF-α, LPS-induced death was due to hepatic necrosis with associated inflammation. We hypothesized that IKKβKA expression under these conditions might protect animals by reducing proinflammatory pathways. In contrast, we hypothesized that in the absence of TNF-α, such proinflammatory pathways played a minor role in LPS-induced hepatic apoptosis in the setting of IKKβKA expression. To directly assess the extent to which proinflammatory pathways might differ in response to the TNF-α and IKKβ activation status of the liver, we evaluated the serum levels of 10 cytokines in the four experimental animal groups (Fig. 7). We have categorized the cytokine responses into two major groups.

The first group of cytokines included those whose levels were reduced by IKKβKA expression in a TNF-α-dependent manner. MIP-1α, IL-1α, IL-1β, and IL-10 were examples of cytokines reduced in TNF-$\alpha^{+/-}$ mice following IKKβKA expression. These same cytokines had little or no change in TNF-$\alpha^{-/-}$ animals as a result of IKKβKA expression. MIP-1α is a component of the acute phase response to LPS (31). IL-1α is a proinflammatory cytokine secreted by macrophages that may contribute to mortality in sepsis (20). The overall levels of MIP-1α and IL-1α were significantly lower in TNF-$\alpha^{-/-}$ mice, while both IL-1β and IL-10 were induced in a similar manner in both TNF-$\alpha^{+/-}$ and TNF-$\alpha^{-/-}$ mice. IKKβKA-dependent reduction in all of these cytokines only reached significance on the TNF-$\alpha^{+/-}$ background (see asterisks in Fig. 7).

A second group of cytokines included those that had no significant IKKβKA-induced change, regardless of the TNF-α genotype. This group included IL-6, IL-12, IFN-γ, KC, GM-CSF, and RANTES. Some of these cytokines (IL-6 and IL-12)

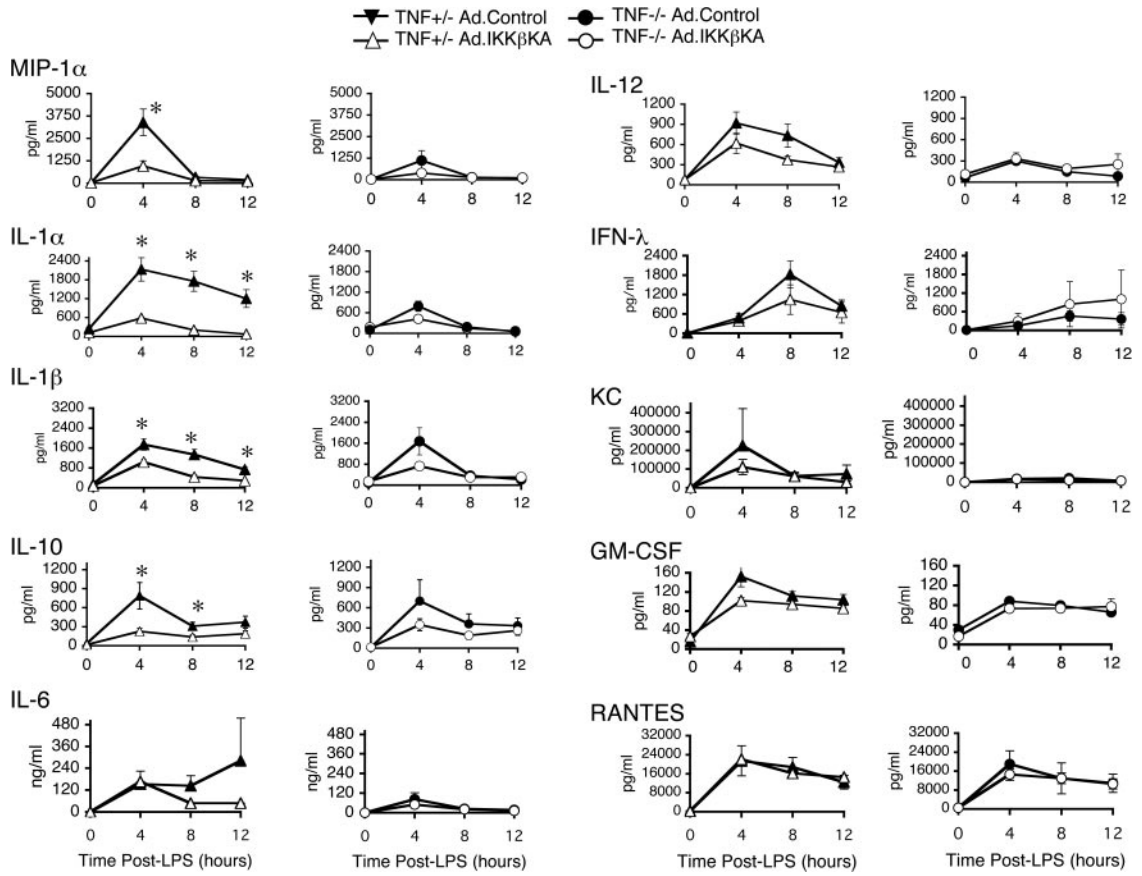

Fig. 7. Hepatic IKKβKA expression reduces LPS-induced inflammatory cytokines in a TNF-α-dependent fashion. Mice were infected with adenoviral constructs, as indicated at the top of each panel 2 wk before LPS administration. Sera were collected at different time points after LPS injections, as indicated on the *x*-axis. Cytokine concentrations shown were detected by multiplex Bio-Rad assay as described in MATERIALS AND METHODS. Each point represents a mean ± SE of $n \geq 3$ independent data points. *Statistically significant differences using a nonpaired *t*-test ($P < 0.05$).

exhibited trends of decreased expression in the Ad.IKKβKA-infected TNF-$\alpha^{+/-}$ mice. Both IL-6 and IL-12 are proinflammatory cytokines and in certain instances play a pivotal role in the pathogenesis of septic shock (8, 41). In addition, the levels of IL-6, IL-12, and KC were all significantly lower in the TNF-$\alpha^{-/-}$ mice, regardless of the vector used for hepatic infection. IFN-γ is secreted by natural killer cells in response to LPS (37), and treatment of mice with anti-TNF-α-neutralizing antibodies reduces serum IFN-γ levels by 20% post-LPS challenge (12). This partial dependence of IFN-γ induction on TNF-α correlated with significantly lower serum levels of IFN-γ in TNF-$\alpha^{-/-}$ mice. KC is a C-X-C chemokine that serves to recruit neutrophils to local sites of inflammation (16). KC levels were increased in TNF-$\alpha^{+/-}$ mice after LPS administration but showed no increase in the TNF-$\alpha^{-/-}$ mice.

To provide supportive evidence for the different death phenotypes seen in TNF-$\alpha^{+/-}$Ad.Control-infected (necrosis) and TNF-$\alpha^{-/-}$Ad.IKKβKA-infected (apoptotic) animals, we compared cytokine profiles between these two experimental groups (Fig. 8). We reasoned that such a direct comparison would provide support for systemic inflammation being the cause of necrotic death in TNF-$\alpha^{+/-}$Ad.Control-infected animals, a phenotype not observed in the caspase-mediated apoptotic death of TNF-$\alpha^{-/-}$Ad.IKKβKA-infected mice. MIP-1α, IL-1α, IL-1β, IL-12, and GM-CSF were all significantly lower in TNF-$\alpha^{-/-}$Ad.IKKβKA-infected mice compared with TNF-$\alpha^{+/-}$Ad.Control-infected animals following LPS administration (Fig. 8). These cytokines are proinflammatory, and higher levels support inflammation as being the primary cause of necrotic death in TNF-$\alpha^{+/-}$Ad.Control-infected animals. In summary, these data support a more active LPS-induced inflammation in TNF-α-competent animals leading to necrotic hepatic death. In contrast, they also suggest that inflammation plays less of a role in apoptotic hepatic death induced by IKKβ inhibition in the absence of TNF-α.

JNK phosphorylation following LPS challenge is dynamically regulated in an IKKβ- and TNF-α-dependent manner. JNK activation has been reported to play a proapoptotic role after hepatic insult, as well as contributing to inflammatory responses (18, 39, 44). We hypothesized that differential regulation of JNK might explain how IKKβKA expression influences necrotic vs. apoptotic hepatic cell death in a TNF-α-dependent fashion after LPS challenge. Therefore, we assessed hepatic JNK activation in TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/-}$ mice following LPS challenge and infection with Ad.IKKβKA or Ad.Control vectors. These studies demonstrated sustained levels of hepatic phospho-JNK (pJNK) at 1–4 h post-LPS administration in control vector-infected TNF-$\alpha^{+/-}$ mice, followed by a significant ($P < 0.05$) decline between 4–8 h. In contrast, hepatic expression of IKKβKA in TNF-$\alpha^{+/-}$ mice led to a significant ($P < 0.05$) decline in pJNK immediately following LPS administration (2–4 h) followed by a return to baseline

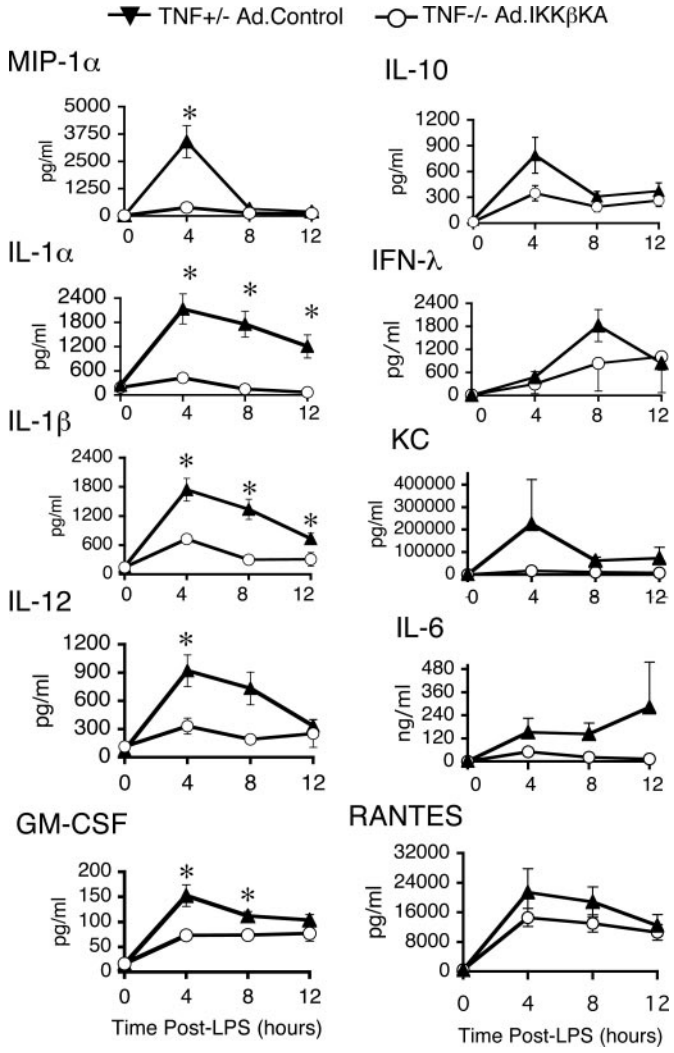

Fig. 8. Differences in cytokine profiles between necrotic and apoptotic LPS-induced death phenotypes dependent on TNF-α. Mice were infected with adenoviral constructs as indicated at the top of each panel 2 wk before LPS administration. Sera were collected at different time points after LPS injections, as indicated on the *x*-axis. Cytokine concentrations shown were detected by multiplex Bio-Rad assay, as described in MATERIALS AND METHODS. Each point represents a mean ± SE of $n \geq 3$ independent data points. *Statistically significant differences using a nonpaired *t*-test ($P < 0.05$).

levels by 8 h (Fig. 9*A*). These results suggest that attenuating NF-κB activation following LPS administration reduces levels of activated JNK in the liver. In contrast, hepatic IKKβKA expression did not alter the levels of pJNK in the TNF-$\alpha^{-/-}$ mice compared with Ad.Control-infected mice following LPS challenge (Fig. 9*A*). Interestingly, JNK activation in the absence of TNF-α remained low in both vector groups immediately following LPS challenge and significantly ($P < 0.05$) rose between 2 and 8 h after LPS challenge. Hence, in contrast to our original hypothesis, changes in the activated state of JNK could not explain IKKβ-dependent differences in hepatic apoptosis between TNF-$\alpha^{+/-}$ and TNF-$\alpha^{-/-}$ mice. However, a reduction in TNF-α following LPS challenge (either through IKKβKA-mediated inhibition of NF-κB in TNF-α-competent mice or through knockout of the TNF-α gene), did lead to lower levels of pJNK initially following injury in both mouse model systems tested. These findings suggest that phosphorylation of JNK following LPS challenge is likely directly influenced by TNF-α.

GADD45β has been reported to play an antiapoptotic role by inhibiting MKK7-mediated activation of JNK (32). GADD45β is also involved in the regulation of cell cycle progression and apoptosis and is regulated at the transcriptional level by NF-κB. To this end, we sought to investigate whether changes in GADD45β levels might explain differences in predisposition to hepatocellular apoptosis in our models of endotoxemia. Quantitative Western blot analysis of livers from TNF-$\alpha^{+/-}$ animals demonstrated no significant differences in the profile of GADD45β expression between the two Ad.IKKβKA or Ad.Control vectors groups following LPS-challenge (Fig. 9*B*). In contrast, Ad.IKKβKA-infected TNF-$\alpha^{-/-}$ mice demonstrated significantly elevated baseline levels of GADD45β (as compared with Ad.Control-infected animals on the same genetic background), which remained elevated following LPS-treatment. The pattern of GADD45β expression in Ad.Control-vector treated TNF-$\alpha^{-/-}$ mice was significantly different from that seen in Ad.IKKβKA-infected mice (Fig. 9*B*); baseline levels of GADD45β protein were low for the first 4 h following LPS challenge and significantly rose at later time points. We hypothesize that the elevated baseline GADD45β is a compensatory mechanism attempting to counteract a heightened predisposition to apoptosis in the absence of TNF-α. Hence, although changes in GADD45β do not appear to play a direct role in LPS-induced apoptosis seen in IKKβKA expressing TNF-$\alpha^{-/-}$ mice, the elevated baseline of GADD45β expression in these mice suggests that IKKβKA-dependent hepatic preconditioning in the absence of TNF-α may predispose the liver to LPS-induced apoptosis. In support of this hypothesis, baseline levels of cIAP2 were elevated in Ad.IKKβKA-infected TNF-$\alpha^{-/-}$ animals compared with Ad.Control-infected animals (data not shown), also suggesting that Ad.IKKβKA-infection alone may trigger an antiapoptotic response in the absence of TNF-α. cIAP2 is regulated by NF-κB and has been reported to inhibit the activation of caspase 3, 7, and 9 (10).

DISCUSSION

Our findings demonstrate that inhibiting hepatic IKKβ can protect mice from endotoxemia by reducing serum TNF-α and other proinflammatory cytokines that lead to an inflammation-based necrotic death of the liver. Such findings suggest that TNF-α and NF-κB play predominantly detrimental roles in endotoxin-induced liver damage. However, studies in TNF-α-knockout mice suggest that the roles of TNF-α and NF-κB activation during endotoxemia are considerably more complex. In the absence of TNF-α, hepatic apoptosis was significantly increased in the presence of IKKβKA expression. These findings suggest that TNF-α may actively modulate NF-κB-independent survival pathways to prevent apoptosis and that only in the absence of TNF-α does the NF-κB protective pathway become the predominant mode of hepatocyte survival.

Reduction of LPS-induced liver injury by hepatic IKKβKA expression in wild-type mice was a surprising finding as severe liver degeneration and enhanced apoptotic responses to TNF-α have been observed in knockout mice and fibroblasts, respectively, lacking IKKβ or NEMO/IKKγ (23, 24, 35). Hence, NF-κB functions as an antiapoptotic factor during embryonic

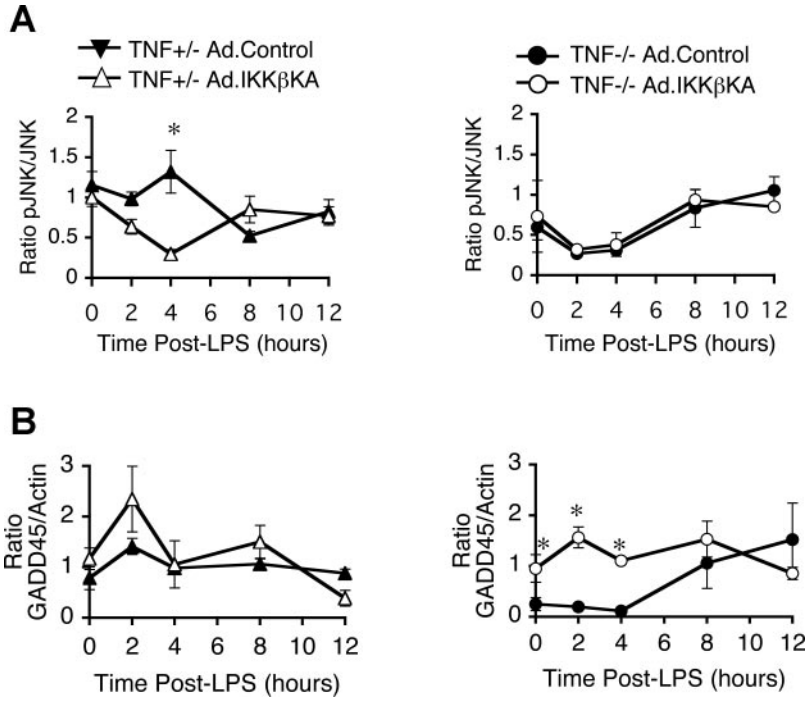

Fig. 9. Inhibition of IKKβ influences JNK phosphorylation and GADD45β expression in a TNF-α-dependent manner. TNF-$\alpha^{+/-}$ or TNF-$\alpha^{-/-}$ nude animals were infected with either Ad.Control or Ad.IKKβKA constructs, 2 wk before IP LPS challenge at 20 μg/gbw. Liver tissue was harvested at different time points after LPS injection, and ratios of pJNK/JNK (*A*) were calculated. The ratio of each point represents a mean ± SE of $n \geq 3$ independent data points. Ratios of GADD45β/actin (*B*) were calculated. The ratio of each point represents a mean ± SE of $n \geq 3$ independent data points. *Statistically significant differences between time-matched data sets using a Student's *t*-test ($P < 0.05$).

liver development and a functional IKKβ subunit is required for this process. Our findings demonstrating opposite effects of hepatic IKKβKA on survival following LPS challenge or PH support a context-dependent protective role for NF-κB during these two types of liver injury. The influence of NF-κB on hepatocellular regeneration during the course of endotoxemia is presently unclear. However, TNF-α has been shown to play a critical role in priming hepatocellular regeneration in the setting of PH (6, 21, 43), and these effects may also play a role in endotoxemia.

One previous study has evaluated the involvement of hepatic IKKβ in LPS-induced injury using an albumin promoter-driven CRE knockout of IKKβ specifically in hepatocytes (28). Interestingly, ablation of IKKβ in this study did not alter serum TNF-α levels or hepatic injury (as judged by serum ALT levels) in response to systemic LPS administration. The authors concluded that inhibition of NF-κB activation is not sufficient for the induction of liver failure in the presence of high levels of circulating TNF-α. This previous study has differences from and similarities to our current work that are worth noting. First, recombinant adenovirus targets IKKβ inhibition in a wider range of hepatic cell-types than just hepatocytes. At the doses of vector used, we have previously shown that the majority of hepatocytes and Kupffer cells of the liver effectively express transgenes (47). Because Kupffer cells of the liver are a predominant source of TNF-α after LPS administration (27), this may explain why in our studies IKKβKA expression effectively reduced serum TNF-α post-LPS administration to wild-type mice. Second, in TNF-α-knockout mice, we have shown that hepatic IKKβKA expression induces apoptotic liver failure. These findings suggest that TNF-α in addition to being harmful, also plays a protective role in preventing apoptosis. Hence, the proinflammatory and antiapoptotic functions of NF-κB appear to be uniquely regulated by TNF-α in the liver in response to LPS.

In wild-type mice, hepatic IKKβKA expression decreased serum levels of several proinflammatory and anti-inflammatory cytokines, suggesting that the overall extent of both inflammation and the resolution of inflammation were abrogated by lower TNF-α levels. Hence, reducing TNF-α production by hepatic IKKβKA expression appears to tilt the balance in favor of hepatic survival by reducing inflammation and necrosis, while retaining antiapoptotic pathways influenced by TNF-α. Enhanced hepatic caspase-3-mediated apoptosis in TNF-α-knockout mice expressing IKKβKA suggests that TNF-α influences the context in which NF-κB functions to prevent apoptosis.

Recently, it has been shown that changes in JNK activation affect apoptotic fates. Subtle changes in the relative activities of NF-κB and JNK1 can have dramatic effects on cell survival and the pattern of JNK activation (prolonged vs. acute) can significantly influence apoptosis (7, 25, 26). In the presence of TNF-α, our studies demonstrated a clear regulation of JNK by IKKβ; expression of IKKβKA in wild-type animals clearly attenuated pJNK levels following LPS administration. Given that JNK phosphorylation has been shown to promote inflammatory responses (39, 44), attenuation of pJNK by IKKβKA expression in wild-type mice (i.e., TNF-$\alpha^{+/-}$) substantiates findings of a reduced proinflammatory cytokine profile (including TNF-α) following LPS challenge. Interestingly, in TNF-α-knockout mice, lower levels of pJNK immediately following LPS administration were independent of IKKβ activity (i.e., seen in both Ad.Control and Ad.IKKβKA-infected groups). This suggests that TNF-α may be responsible for maintaining pJNK levels immediately following LPS injury, a hypothesis supported by the ability of IKKβKA expression to attenuate both TNF-α and pJNK levels in wild-type animals. However, given that the profile of pJNK changes were similar in both vector-treated TNF-$\alpha^{-/-}$ groups of mice (Ad.Control-infected and Ad.IKKβKA-infected), changes in JNK phosphorylation

cannot account for observed differences in NF-κB-dependent apoptosis.

In conclusion, this is the first direct demonstration of a protective role for TNF-α in a model of endotoxin induced liver damage. In the presence of TNF-α, LPS injury is reduced by inhibiting NF-κB activation in the liver, which leads to a reduction in serum TNF-α and subsequent proinflammatory injury that drives hepatic necrosis. In the absence of TNF-α, host survival is dependent on hepatic NF-κB that protects the liver from apoptosis. Under physiological conditions, constitutive expression of cytoprotective proteins confers resistance to LPS-induced apoptosis (2), in addition to the inducible protective pathway. We hypothesize that these constitutive cytoprotective pathways protect the liver from LPS-induced apoptosis in TNF-$\alpha^{+/-}$Ad.IKKβKA-infected animals. Our findings suggest that such cytoprotective pathways are TNF-α-dependent, and that in the absence of TNF-α, the inducible protective NF-κB pathway becomes the predominant survival pathway in the liver. Thus therapies aimed at reducing TNF-α and/or NF-κB in endotoxemia must carefully weigh the detrimental and beneficial roles of both factors in the liver.

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